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13. Abstract ***(Maximum 200 Words)*** Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptors. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) and E2 ubiquitin-conjugating enzyme, UbcH7 as coactivators of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP and UbcH7 may contribute to the development of breast cancer. We have examined this possibility by studying the expression patterns of E6-AP, UbcH7 and estrogen receptor-alpha (ER) in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have correlated the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies. Todate, we have examined 56 advanced stage human breast cancer biopsy samples for the expression profile of E6-AP, UbcH7 and ER. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples. Presently, we are studying the expression profile of E6-AP, UbcH7 and ER in early and intermediate stage tumors. Another goal of this project is to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, E6-AP and UbcH7. In order to achieve this goal, we have already constructed the expression vectors for stable cell lines. Our preliminary data suggest that these vectors produce biologically functional coactivator proteins, E6-AP and UbcH7. Presently, we are in the process of generating the stable cell lines that will stably overexpress E6-AP and UbcH7.

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Introduction

Breast cancer is the leading cause of death in American women. It is anticipated that one woman out of ten will develop breast cancer at some point during her life (Nicholson 1979; Nicholson et al. 1986; Horwitz 1994; Nicholson et al. 1995; Parker et al. 1997; Morris et al. 2001). Although in recent years significant progress has been made in detection and treatment of the disease, much of the molecular basis of the disease remains unknown. This fact highlights the need to identify and understand the molecular basis associated with breast cancer development and progression.

Steroid hormones, estrogen and progesterone, play important role in the development and progression of breast cancer (Benner et al. 1988; Clarke et al. 1989; Clarke et al. 1992; Elledge et al. 2000). Estrogens and progesterones exert their biological effects on target tissues through intracellular receptor proteins, the estrogen (ER) and progesterone (PR) receptors (O'Malley 1990; Tsai and O'Malley 1994; Hatina and Reischig 2000). These receptors contain common structural motifs which include a less well conserved amino-terminal activation function (AF-1) that effects transcription efficiency, which has the hormone-independent activation function; a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determine target gene specificity; and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2); the region mediates the hormone-dependent activation function of the receptors (O'Malley 1990; Tsai and O'Malley 1994; Hatina and Reischig 2000).

In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT) and ATPase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC) (Horwitz et al. 1996; McKenna et al. 1998; McKenna 1999; Chen 2000). These events are followed by up- or down-regulation of target gene expression.

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. Prior to their identification, coactivators were predicted to exist based upon experiments, which showed that different receptors compete for a limiting pool of accessory factors required for optimal transcription. Stimulation of one receptor resulted in trans-repression of another receptor, indicating the depletion of a common coactivator pool (Bocquel et al. 1989; Meyer et al. 1989; Shemshedini et al. 1992). A number of coactivators have been cloned to date, including SRC-1 (Onate et al. 1995), TIF2 (GRIP1) (Hong et al. 1996; Voegel et al. 1996; Hong et al. 1997; Voegel et al. 1998), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (Anzick et al. 1997; Chen et al. 1997; Li et al. 1997; Takeshita et al. 1997; Torchia et al. 1997), PGCs (Puigserver et al. 1998), SRA (Lanz et al. 1999), CBP (Ikonen et al. 1997; Aarnisalo et al. 1998; Fronsdal et al. 1998), **E6-associated protein (E6-AP), and ubiquitin conjugating enzymes such as UbcH5B, UbcH7 and Ubc9** (Nawaz et al. 1999b; Poukka et al. 1999; Poukka et al. 2000) and this list is growing rapidly day by day.

Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (Pugh and Tjian 1992; Tjian and Maniatis 1994). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities that may contribute to their ability to enhance receptor mediated transcription; SRC-1, p300/CBP, and ACTR (RAC3/AIB1) possess a histone acetyl transferase, HAT, activity (Ogryzko et al. 1996; Anzick et al. 1997; Li et al. 1997; Spencer et al. 1997; McKenna et al. 1998; Collingwood et al. 1999; Chen 2000) and members of SWI/SNF complex contain an ATPase activity (Dunaief et al. 1994; Muchardt et al. 1996; Wang et al. 1996; Reyes et al. 1997). Ligand-activated receptors are thought to bring HAT and ATPase activities containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin associated factors and catalyzing the uncoupling of ionic interactions between histones and their substrate DNA (Dunaief et al. 1994; Muchardt et al. 1996; Ogryzko et al. 1996; Wang et al. 1996; Yang et al. 1996; Reyes et al. 1997; Spencer et al. 1997). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological responses to hormones (Xu et al. 1998; McKenna 1999; Leo and Chen 2000; Xu et al. 2000). The level of coactivator expression is critical in determining the activity of the receptor in target tissues and variations in hormone responsiveness seen in the population may be due to differences in coactivator levels.

It is accepted that coactivators either possess or bring HAT and ATPase activities to the promoter region of the target genes and presumably manifest part of their in vivo coactivation functions through these enzymatic activities (Dunaief et al. 1994; Muchardt et al. 1996; Ogryzko et al. 1996; Wang et al. 1996; Reyes et al. 1997; Spencer et al. 1997). Recent identification of the enzymes of the ubiquitin-proteasome and ubiquitin-like pathways as coactivators by my own laboratory and others added a new twist to the coactivator field. These studies suggest that the ubiquitin-conjugating enzymes, UbcH5B, UbcH7 and Ubc9 and the E3 ubiquitin-protein ligases, E6-AP and RPF1/RSP5, interact with members of the steroid hormone receptor superfamily including ER and PR and modulate their transactivation functions (Imhof and McDonnell 1996; McKenna et al. 1998; Nawaz et al. 1999b; Poukka et al. 1999; Poukka et al. 2000). Similarly, another coactivator protein, yeast SUG1, an ATPase subunit of the 26S-proteasome complex also interacts with and modulates steroid hormone receptor function (Fraser et al. 1997; Makino et al. 1997; Masuyama and MacDonald 1998). Instead of HAT activity, this group of coactivators possesses other enzymatic activities such as ubiquitin conjugation, ubiquitin ligation and protease activities. However, a common theme between the two groups of coactivators is that both possess some sort of enzymatic activity.

As mentioned above, my laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. We have cloned an E3 ubiquitin-protein ligase, E6-AP as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR) (Nawaz et al. 1999b). E6-AP was previously identified as a protein of 100 kDa, present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically

interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (Huibregtse et al. 1991; Huibregtse et al. 1993). As mentioned above, E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases. E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin system (Scheffner et al. 1993; Huibregtse et al. 1995b; Huibregtse et al. 1995a). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and many E2 ubiquitin conjugating enzymes (UBCs). The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond. In some cases, ubiquitin is transferred directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (Ciechanover 1994; Ciechanover and Schwartz 1994).

The E2 ubiquitin conjugating enzymes of the ubiquitin pathway, UbcH5B and UbcH7 (UBCs) also act as coactivators of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner. Treatment of mammalian cells with the proteasome inhibitor, MG132, which specifically blocks the protease activity of the proteasome, blocks ER degradation. This suggests that ER protein is degraded through the ubiquitin-proteasome pathway (Nawaz et al. 1999a). In addition, our results also suggest that the estrogen-dependent degradation of ER correlates with hormone-dependent ER activation because MG132 not only blocks ER protein degradation but also block its activation. Our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the UBCs, UbcH5B and UbcH7 and ubiquitin-proteasome pathway (Nawaz et al. 1999a). These observations raise the question as to why ubiquitin pathway enzymes and ubiquitin-dependent protein degradation are linked to steroid hormone receptor activation. Considering that the transcriptionally active receptor is associated with a diverse group of proteins and forms a preinitiation complex, it is possible that subsequent to receptor activation of transcription, ubiquitin mediated degradation of the receptor may be a mechanism which dissociates the preinitiation complex. It could be necessary to dissociate the preinitiation complex through targeted protein degradation since the synergistic interactions of multiple transcription factors may make passive dissociation of hormone and coactivators impossible or time consuming. Additionally, it is possible that hormone-induced receptor degradation serves to control physiological responses to steroid hormones ultimately limiting the expression of steroid-responsive genes.

It has been shown that altered expression of one nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancer. Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function. Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways (Anzick et al. 1997). Recenly, it has also been shown that another steroid receptor coactivator, SRA, is also elevated in breast tumors (Murphy et al. 2000). Furthermore, we have also shown that E6-AP is

overexpressed 2.5-4.5 fold in 90-95% of tumors using a mouse mammary model of multistage tumorigenesis. E6-AP is overexpressed only in tumors but not in the intermediate steps of tumorigenesis (Sivaraman et al. 2000).

The purpose of this research is to explore the possibility that the altered expression of UbcH5B, UbcH7 and E6-AP may contribute to the development of breast cancer. In the original proposal, we proposed to explore this possibility by studying the expression profile of UbcH5B, UbcH7, and ER in various breast cancer cell lines and breast tumor biopsy samples. We also proposed to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, UbcH5B and UbcH7. We have examined the expression patterns of E6-AP, UbcH7, with that of ER in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have also correlated the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies. Unfortunately, due to the lack of good UbcH5B antibody, we are unable to study the expression profile of UbcH5B. Instead of UbcH5B, we have examined the expression profile of E6-AP in several breast cancer cell lines and breast biopsy tumors. To-date, we have examined 56 advanced stage human breast cancer biopsy samples for the expression profile of E6-AP, UbcH7 and ER. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples. Presently, we are studying the expression profile of E6-AP, UbcH7 and ER in early and intermediate stage tumors. Another goal of this project is to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, E6-AP and UbcH7. In order to achieve this goal, we have already constructed the expression vectors for stable cell lines. Our preliminary data suggest that these vectors produce biologically functional coactivator proteins, E6-AP and UbcH7. Presently, we are in the process of generating the stable cell lines that will stably overexpress E6-AP and UbcH7.

Body

In this original proposal, we hypothesized that ubiquitin-conjugating enzymes, UbcH5B, UbcH7 and an E3 ubiquitin-protein ligase, E6-AP, are important modulators of the steroid hormone receptor-mediated signal transduction pathway, cell growth, and cell cycle control in the context of breast cancer development. In order to test this hypothesis we propose following objectives:

- **Expression analysis of endogenous ubiquitin-conjugating enzymes, UbcH5B and UbcH7, and ER in breast cancer cell lines and human breast tumor biopsies. Then compare the expression patterns of UbcH5B and UbcH7 with that of ER.**
- **Design and development of stable in vitro models of UbcH5B and UbcH7 overexpression in the breast cancer cell lines.**
- **Analysis of the growth properties of stably transfected cell lines that overexpress UbcH5B and UbcH7 and in vivo analysis of tumorigenicity of these stably transfected cell lines in athymic nude mice.**

Expression analysis of endogenous ubiquitin-conjugating enzymes, UbcH5B and UbcH7, and ER in breast cancer cell lines and human breast tumor biopsies. Then compare the expression patterns of UbcH5B and UbcH7 with that of ER.

One of the aims of this proposal is to test the expression of endogenous UbcH5B, UbcH7 and ER in human breast cancer cell lines and human breast tumor biopsies. Then compare the expression profile of UbcH5B and UbcH7 with that of ER. To date we have examined expression levels of UbcH7, E6-AP, and ER in 56 different breast tumors and expression of p53 in 20 different tumors. Additionally, we have also examined the expression profile of UbcH7, E6-AP and ER in different breast cancer cell lines. Due to the lack of the availability of the UbcH5B antibody, we are unable to examine the expression profile of UbcH5B. Furthermore, we were not successful in generating a good UbcH5B antibody. Since these proteins are also targets of the ubiquitin-proteasome pathway, we did not analyze the mRNA levels of UbcH5B. Additionally, UbcH5B and UbcH7 both act as E2 ubiquitin-conjugating enzymes for E6-AP, therefore, we also examined the expression of E6-AP in different breast cancer cell lines and breast tumor biopsies. We found an inverse correlation between the expression of E6-AP and the expression of ER in human biopsy tumor samples, and we did not find any statistically significant correlation between the expression profile of UbcH7 and ER.

Task 1. Expression analysis of UbcH7, ER and E6-AP in different breast cancer cell lines.

A. UbcH7 expression

We have analyzed the expression profile of UbcH7, ER and E6-AP in different breast cancer cell lines such as MCF-7, T47-D, ZR75-1 and MDA-MB-231. As a control we have also examined the expression profile of UbcH7, ER and E6-AP in HeLa (a cervical carcinoma cell line) cells. As shown in Figure 1, HeLa cells express high levels of UbcH7 protein compare to that of different breast cancer cell lines (MCF-7, T47-D, ZR75-1 and MDA-MB-231). Furthermore, in HeLa cells, the UbcH7 expression is both cytoplasmic and nuclear. The expression level of UbcH7 in MCF-7, T47-D, ZR75-1 and MDA-MB-231 is moderate compare to that of HeLa cells. In T47-D cells the expression of UbcH7 is totally nuclear whereas in other breast cancer cell lines, MCF-7, ZR75-1 and MDA-MB-231, weak cytoplasmic staining of UbcH7 was observed, in addition to nuclear staining.

B. ER expression

Since we want to compare the expression profile of UbcH7 with that of ER, we also analyzed the expression of ER-alpha in MCF-7, T47-D, ZR75-1 and MDA-MB-231 cell lines. In this case HeLa cell line was used as a negative control. As shown in Figure 2, HeLa cells are negative for ER expression. Similarly, in the breast cancer cell line, MDA-MB-231, the ER expression was undetectable. However, this cell line expresses UbcH7 at moderate level. In contrast to MDA-MB-231, the MCF-7, T47-D and ZR75-1 lines express both UbcH7 and ER-alpha. The ER expression is nuclear in these cell lines.

C. E6-AP expression

Since UbcH7 act as an E2 ubiquitin-conjugating enzyme for E6-AP and both the UbcH7 and E6-AP act as coactivators for ER, we decided to analyze the expression profile of E6-AP in breast cancer cell lines. As shown in Figure 3, the breast cancer cell lines, MCF-7, T47-D, ZR75-1 and MDA-MB-231 express high levels of E6-AP. The E6-AP expression is both cytoplasmic and

nuclear in MCF-7, ZR75-1 and MDA-MB-231 cell lines. The MDA-MB-231 cell line expresses more E6-AP in nucleus than in the cytoplasm. Similar to UbcH7, the expression of E6-AP in T47-D cells is purely nuclear. In this case HeLa cells were used as a positive control for E6-AP expression.

Task 2. Effect of steroids on the expression of UbcH7 and E6-AP.

It is possible that steroid hormones (estrogens/progesterones) may regulate endogenous expression of UbcH7 and E6-AP in breast cancer cell lines. To test this possibility, MCF-7, a hormone-dependent breast cancer cell line was grown in the medium containing stripped serum for a week. Afterward, cells were grown either in the absence or presence of steroid hormones for 48 hours and the expression patterns of UbcH7 and E6-AP were determined by fluorescent immunocytochemistry. As shown in Figure 4, in MCF-7 cells, estrogen treatment has no significant effect on the expression profile of UbcH7. The UbcH7 expression is identical both in the absence and presence of estradiol. Similarly, progesterone treatment also has no significant effect on the expression levels of UbcH7 in MCF-7 cells (data not shown). Next we ask whether steroids treatment has any effect on the expression levels of UbcH7 in T47-D cells. As shown in Figure 5, estrogen treatment has no effect on the expression levels of UbcH7 in T47-D cells. The expression levels of UbcH7 are identical both in hormone treated and untreated cells. The same is true for progesterone (data not shown). These data suggest that the expression of UbcH7 is not under the control of steroid hormones.

Next we ask whether steroids regulate the expression of E6-AP. To test the effect of estrogen on the expression pattern of E6-AP, MCF-7 cells were grown in the medium containing stripped serum for a week. Then, cells were treated with either estrogen or vehice for 48 hours and the expression patterns of E6-AP was determined by fluorescent immunocytochemistry. Figure 6 suggests that the estrogen treatment have no significant effect on the expression of E6-AP. The E6-AP expression levels are identical both in the presence and absence of hormone. This data suggests that E6-AP regulation is not under the control of steroids.

As a control for these experiments, we also analyzed the effect of estrogens on the expression of PR and ER. It has been established that estrogen upregulate the expression of PR protein and it down regulate the levels of ER in MCF-7 cells (Lonard et al. 2000). As expected, Figure 7 demonstrate that estrogen treatment increases the expression of PR protein. In contrast, estrogen down regulates ER expression.

Task 3. Expression analysis of ER-alpha, UbcH7 and E6-AP in breast tumor samples.

As mentioned above, the ubiquitin pathway enzymes, UbcH7 and E6-AP act as coactivators of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner via the ubiquitin-proteasome pathway. Additionally, our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the UbcH7 and ubiquitin-proteasome pathway (Nawaz et al. 1999a). To explore the possibility that the altered expression of UbcH7 and E6-AP may contribute to the development of breast cancer, we analyzed the expression profile of UbcH7,

E6-AP and ER in 56 advanced stage breast tumor biopsy samples by Western blot analysis. Figure 8 and 12 show the expression profile of ER-alpha in 56 different human tumor samples. 23 (41%) out of 56 breast tumor samples express significant amount of ER-alpha, however, 33 (59%) tumors express no or degraded forms of ER protein. Since we want to correlate the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies, we examined the expression profile of UbcH7 and E6-AP in these tumors. Figure 9 and 12 show the expression profile of UbcH7 in human breast tumor samples. As shown in Figure 9, majority of the tumors expresses UbcH7. Only 21(%) tumors are negative for UbcH7. Furthermore, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples.

Since UbcH7 acts as an E2 ubiquitin-conjugating enzyme for E6-AP, we also examined the expression profile of E6-AP in these human breast tumoe samples. To study the expression profile of E6-AP in human breast tumors we performed Western blot analysis using an E6-AP specific antibody. Figure 10 shows the expression of E6-AP in 56 different tumor samples. The majority (82%) of the tumors expresses E6-AP. Furthermore, we found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant (Figure 12).

It has been demonstrated that E6-AP promotes the degradation of p53 via the ubiquitin degradation pathway. In the brain of E6-AP knockout animals, the protein levels of p53 accumulate compared to those of normal littermates. Therefore, we also analyzed the endogenous expression of p53 protein from breast tumor biopsies. As shown in Figure 11, p53 expression was not detectable in most tumors except tumor number 7, 10, 13 and 15. Furthermore, there was no statistical correlation between the expression profile of E6-AP and p53. Presently, we are analyzing more tumor samples for p53 expression.

Since we found an inverse correlation between the expression profile of ER-alpha and E6-P, we analyzed whether E6-AP and ER expression colocalized in breast cancer cell lines and breast cancer tumors. In order to study the colocalization of ER and E6-AP, we performed fluorescent immunocytochemistry on T47-D cells. As shown Figure 13, the expression of E6-AP colocalized with that of ER (see merge figure). These data suggest that it is possible that in vivo E6-AP may promote the degradation of ER through the ubiquitin-proteasome pathway.

In order to study the expression profile of E6-AP and ER-alpha in normal human breast tissues, we performed fluorescent immunohistochemistry. As shown in Figure 14, in normal human breast tissues, E6-AP and ER-alpha proteins are expressed in separate a compartment of ductal epithelial cells. ER is expressed in the nuclei of the ductal epithelial cells. In contrast, E6-AP is highly expressed in the cytoplasm of the ductal epithelial cells. The merged image shows the separate location of ER and E6-AP.

In normal breast tissues E6-AP and ER are expressed in separate locations then the question arises, how E6-AP promotes ER degradation? It is possible that E6-AP pattern is different in tumors than normal tissue. To test this possibility, we also performed fluorescent immunohistochemistry on tumor tissues. As shown in Figure 15, the E6-AP expression is

nuclear instead of cytoplasmic in tumor tissues. Furthermore, 80% of tumor cells exhibit coexpression of ER and E6-AP. Presently, we are in the process of correlating E6-AP nuclear expression with tumor grade.

Task 4. Generation of the expression plasmids for overexpression of UbcH7 and E6-AP.

To construct the expression plasmids for the overexpression of UbcH7 and E6-AP, cDNAs of UbcH7 and E6-AP were are cloned into the mammalian expression vector pcDNA3.1. These vectors produce his-tagged UbcH7 and E6-AP proteins. In order to confirm the functional activity of the his-tagged UbcH7 and E6-AP, we performed the transient transfection assays in HeLa cells. As shown in Figure 16, that the his-tagged UbcH7 and E6-AP were able to enhance the transactivation functions of PR, suggesting that the his-tagged UbcH7 and E6-AP proteins are biologically functional. Presently, we are in the process of generating the stable cell lines that overexpress UbcH7 and E6-AP proteins.

Statement of work accomplished/in progress

Task 1. Expression analysis of UbcH7, ER, and E6-AP in different breast cancer cell lines. **Accomplished.**
Task 2. Effect of steroids on the expression of UbcH7 and E6-AP. **Accomplished.**
Task 3. Expression analysis of ER-alpha, UbcH7, and E6-AP in breast tumor samples. **In Progress.**
Task 4. Generation of the expression plasmids for overexpression of UbcH7 and E6-AP. **Accomplished.**
Task 5. Development of stable cell lines. **In Progress.**
Task 6. Characterization of stable cell lines. **Not Attempted Yet.**
Task 7. Determination of growth properties of stable cell lines. **Not Attempted Yet.**
Task 8. Determine the tumorigenicity of stably transfected cell lines in athymic nude mice. **Not Attempted Yet.**

Key Research Accomplishments

- Expression analysis of UbcH7, ER, and E6-AP in different breast cancer cell lines has been completed.
- Effect of steroids on the expression of UbcH7 and E6-AP has been studied.
- The expression analysis of ER, UbcH7, and E6-AP has been analyzed.
- Expression profile of E6-AP has been compared with that of ER expression.
- Expression profile of UbcH7 has been compared with that of ER expression.
- Generation of the expression plasmids for overexpression of UbcH7 and E6-AP has been completed.
- The biological activities of His-tagged UbcH7 and E6-AP has been analyzed.

Reportable Outcomes

The ongoing work described here was presented as a poster and an abstract at the Annual Endocrine Society Meeting (June 2001), in Denver Colorado (see appendix 2).

Conclusions

We have successfully analyzed the expression of UbcH7, E6-AP and ER in different breast cancer cell lines. Additionally, we have also examined the effects of steroids on the expression profile of UbcH7, E6-AP and ER. In order to study the expression profile of UbcH7, E6-AP and ER in human breast tumors, we have examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. Furthermore, E6-AP expression is nuclear in tumors whereas in normal human mammary cells E6-AP is cytoplasmic. However, we did not observe any correlation between the expression of UbcH7 and ER. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Appendices

1. Figures 1-16

2. Abstract

Appendix 1

Figures 1-16

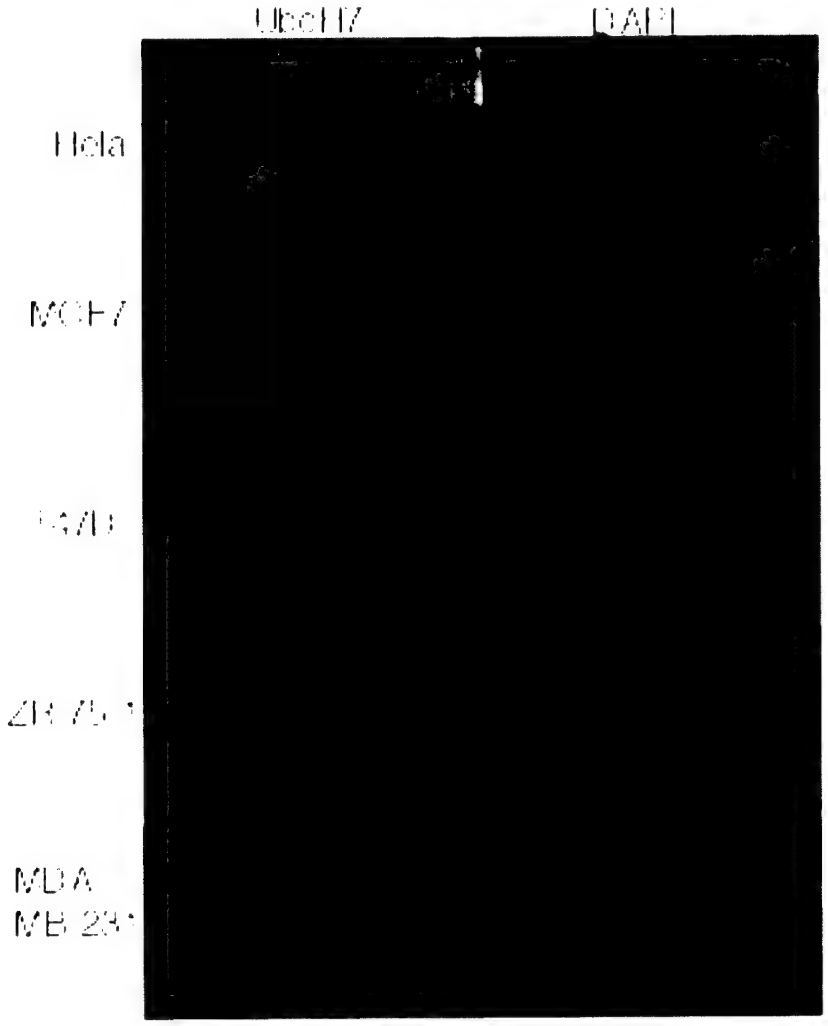

Figure 1: Expression analysis of UbcH7 in different cell lines (Hela, MCF7, T47DZR-75-1 and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and UbcH7 expression was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

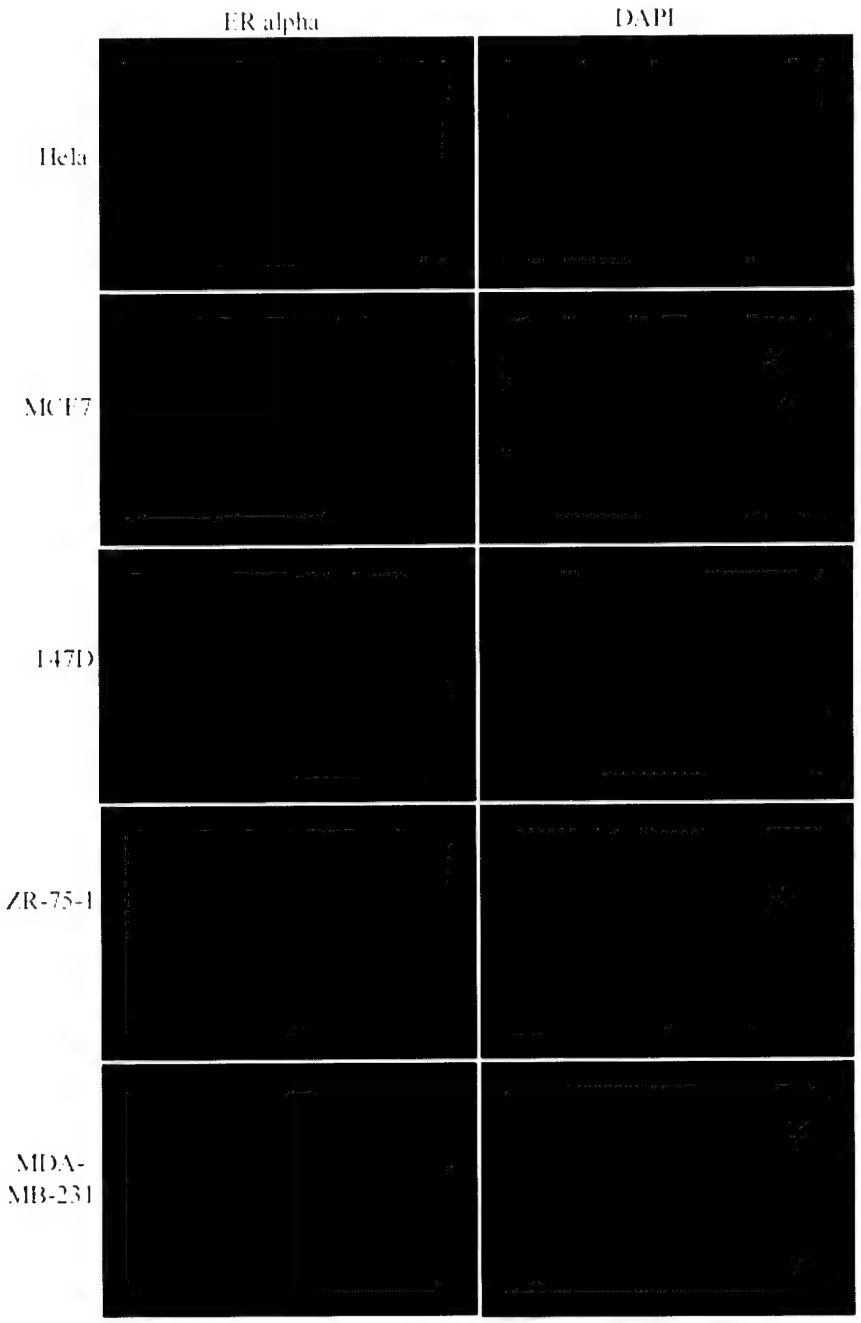

Figure 2: Expression analysis of ER-alpha in different cell lines (Hela, MCF7, T47DZR-75-1and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and ER-alpha expression was analyzed by fluorescent immunocytochemistryusing an anti-ER-alpha antibody. Positive signal for ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. ER-alpha, ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

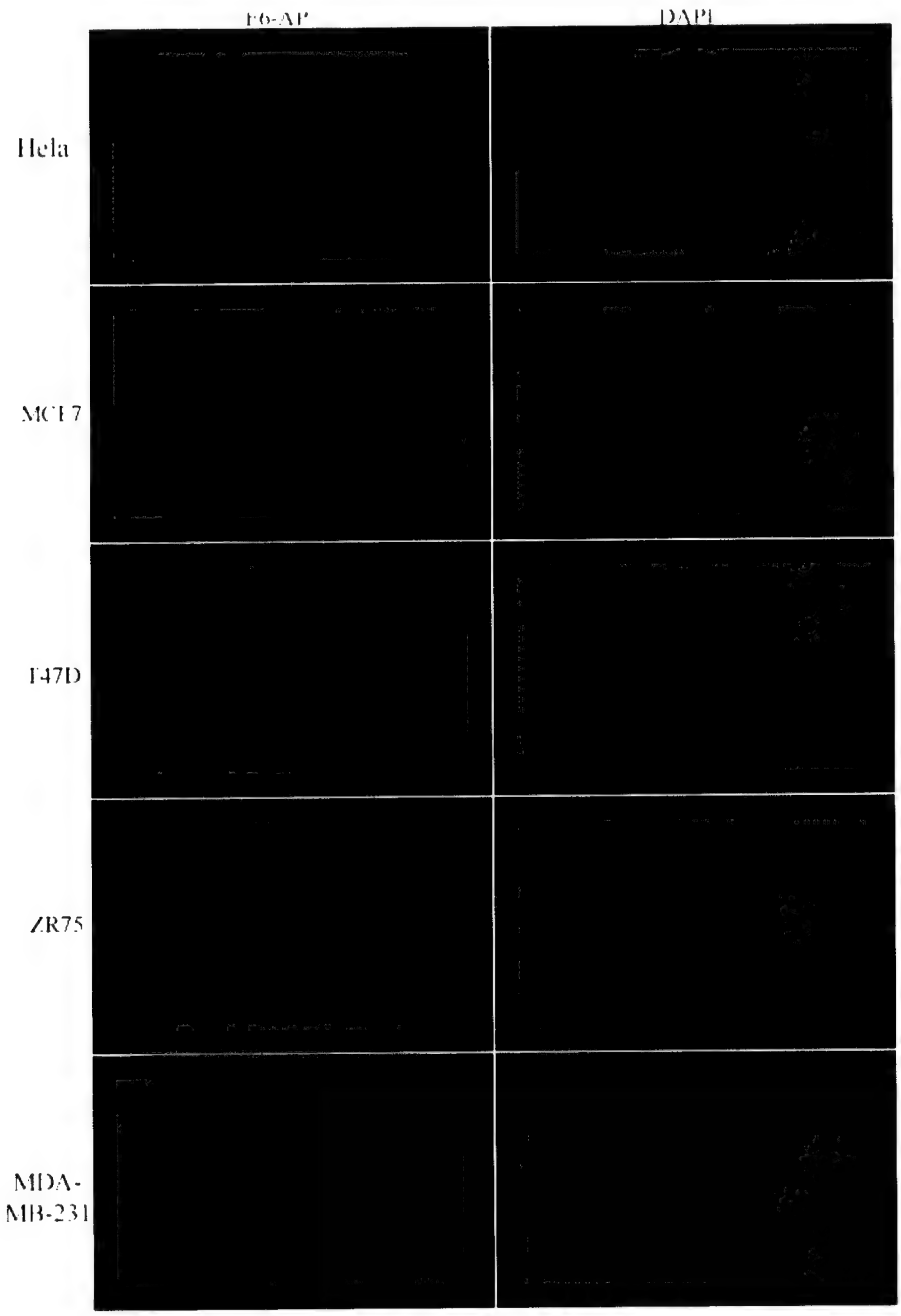

Figure 3: Expression analysis of E6-AP in different cell lines (Hela, MCF7, T47DZR-75-1and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and E6-AP expression was analyzed by fluorescent immunocytochemistryusing an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAP1 staining. E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

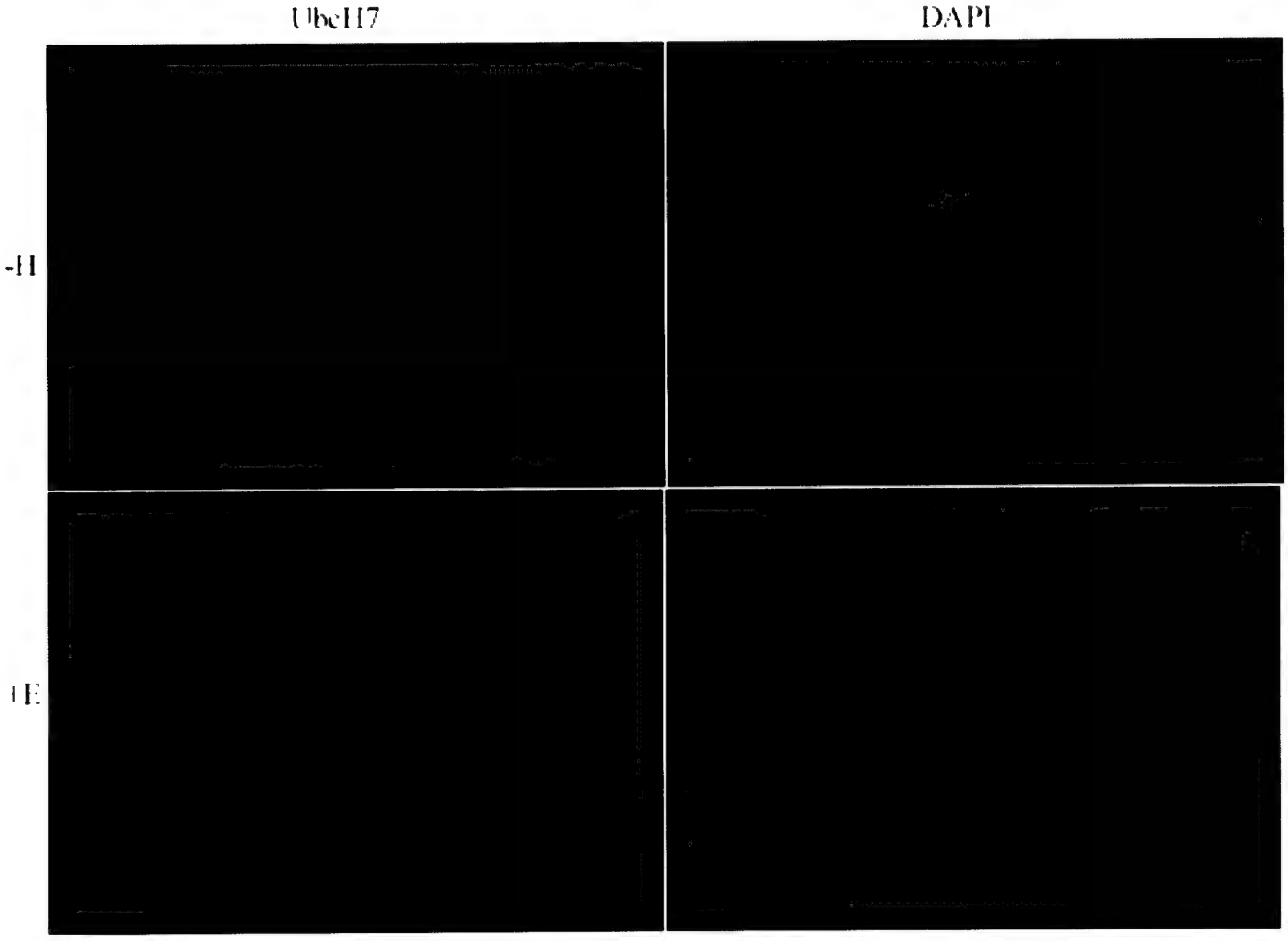

Figure 4: Effect of estrogen on the expression of UbcH7 in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous UbcH7 was analyzed by fluorescent immunocytochemistryusing an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

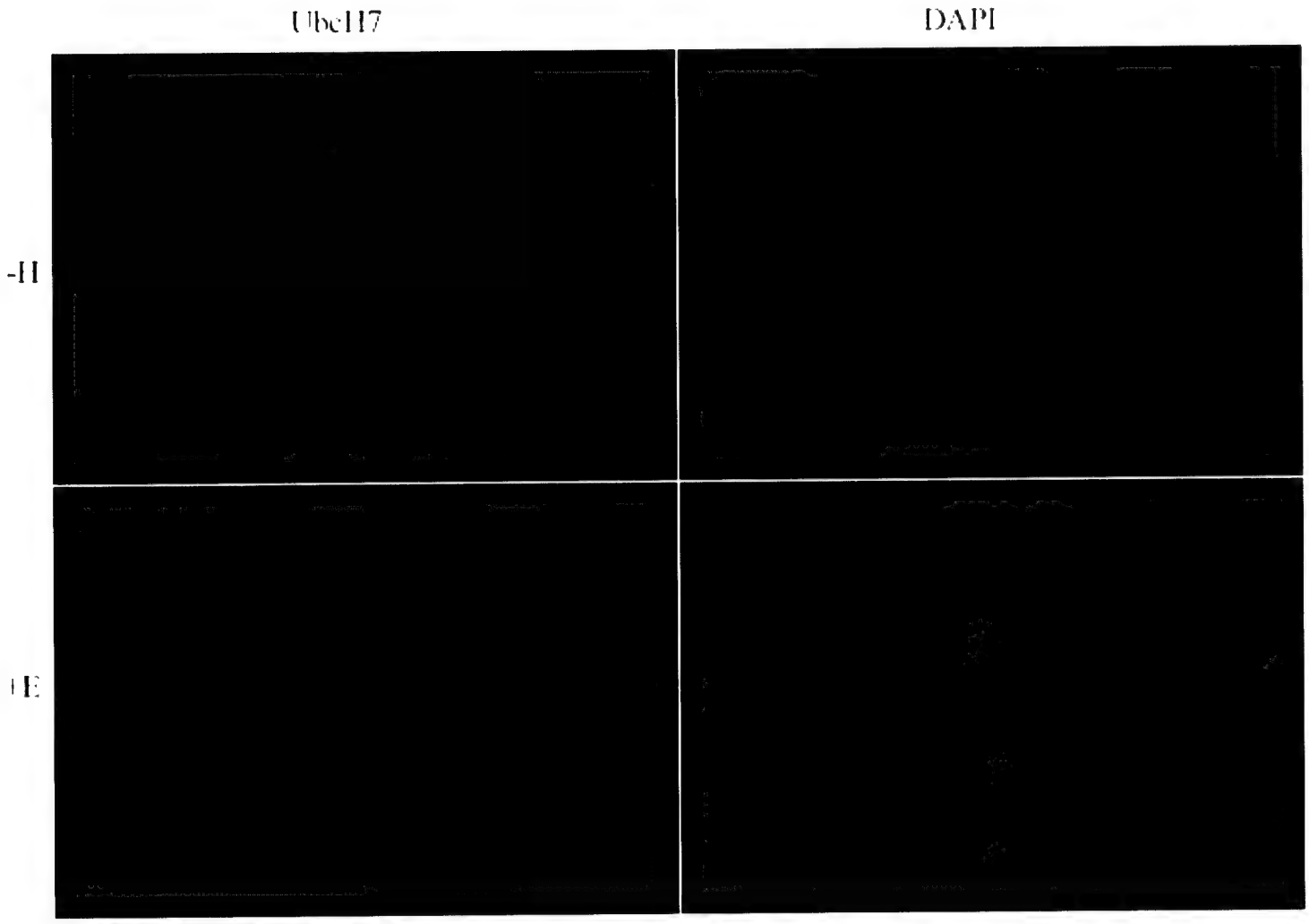

Figure 5: Effect of estrogen on the expression of UbcH7 in T47D cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous UbcH7 was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

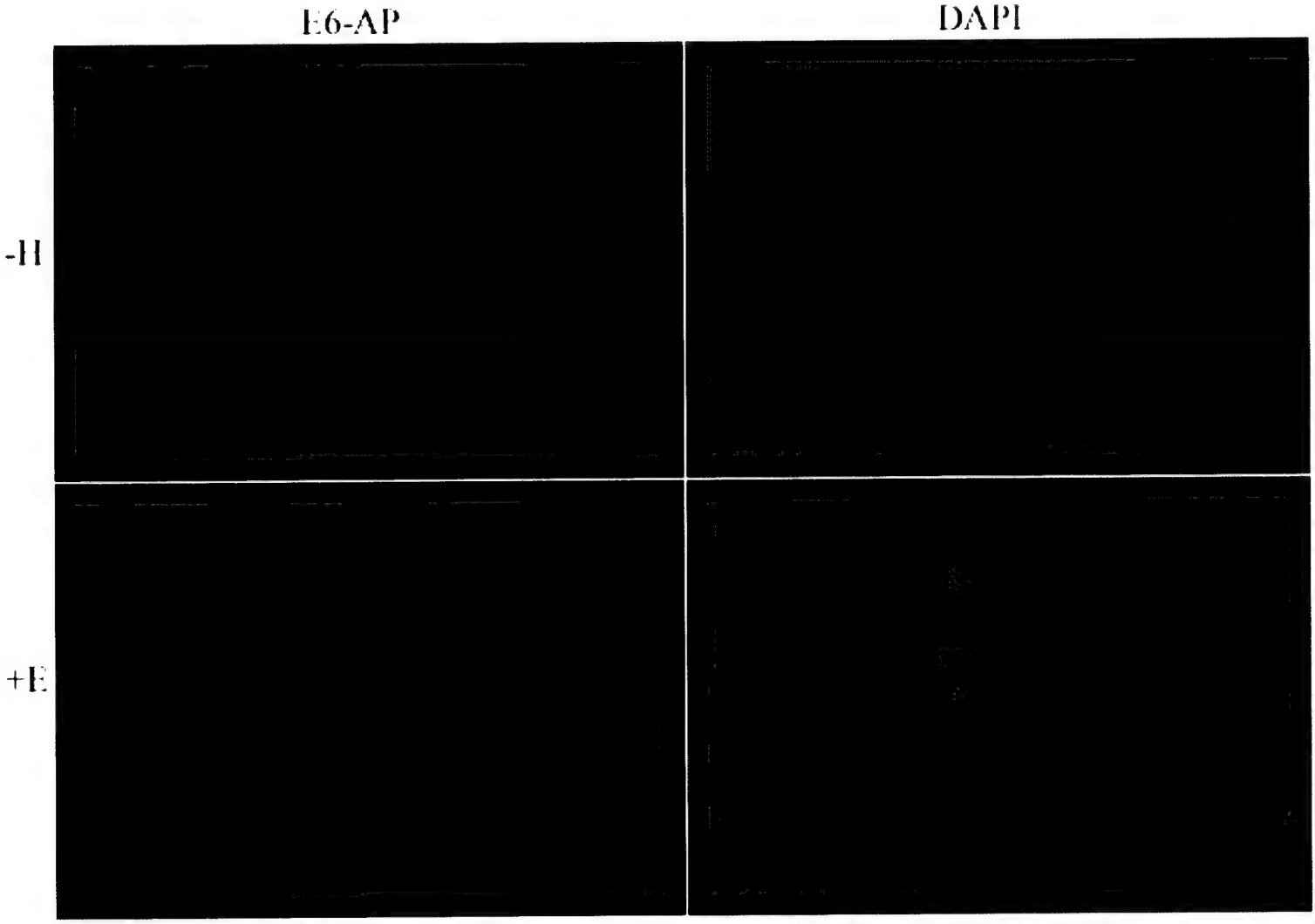

Figure 6: Effect of estrogen on the expression of E6-AP in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous E6-AP was analyzed by fluorescent immunocytochemistryusing an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAP1 staining. E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

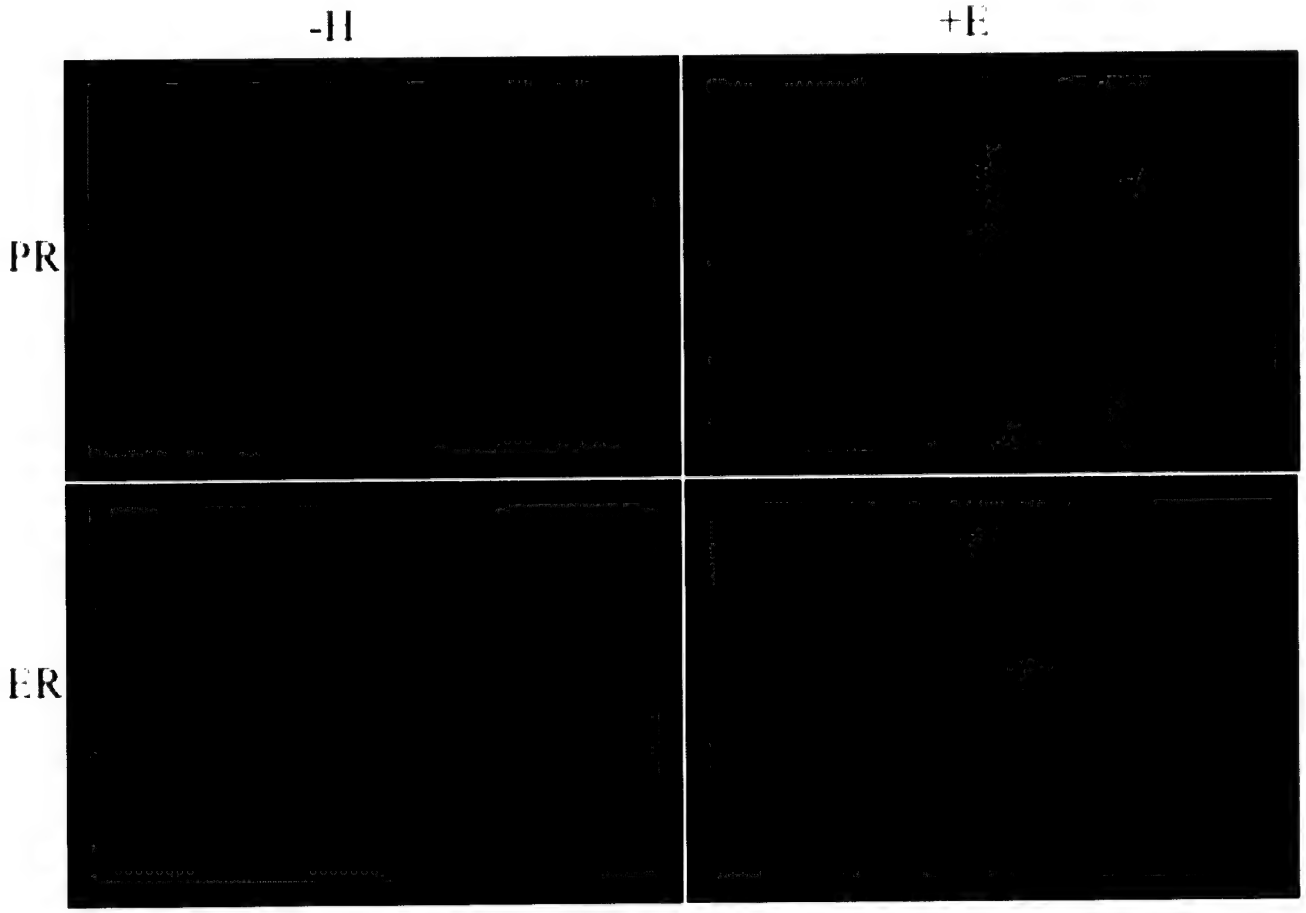

Figure 7: Effect of estrogen on the expression of PR and ER-alpha in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous PR and ER-alpha was analyzed by fluorescent immunocytochemistryusing anti-PR and anti-ER-alpha antibodies. Positive signal for PR and ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. PR, PR expression profile; ER, ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

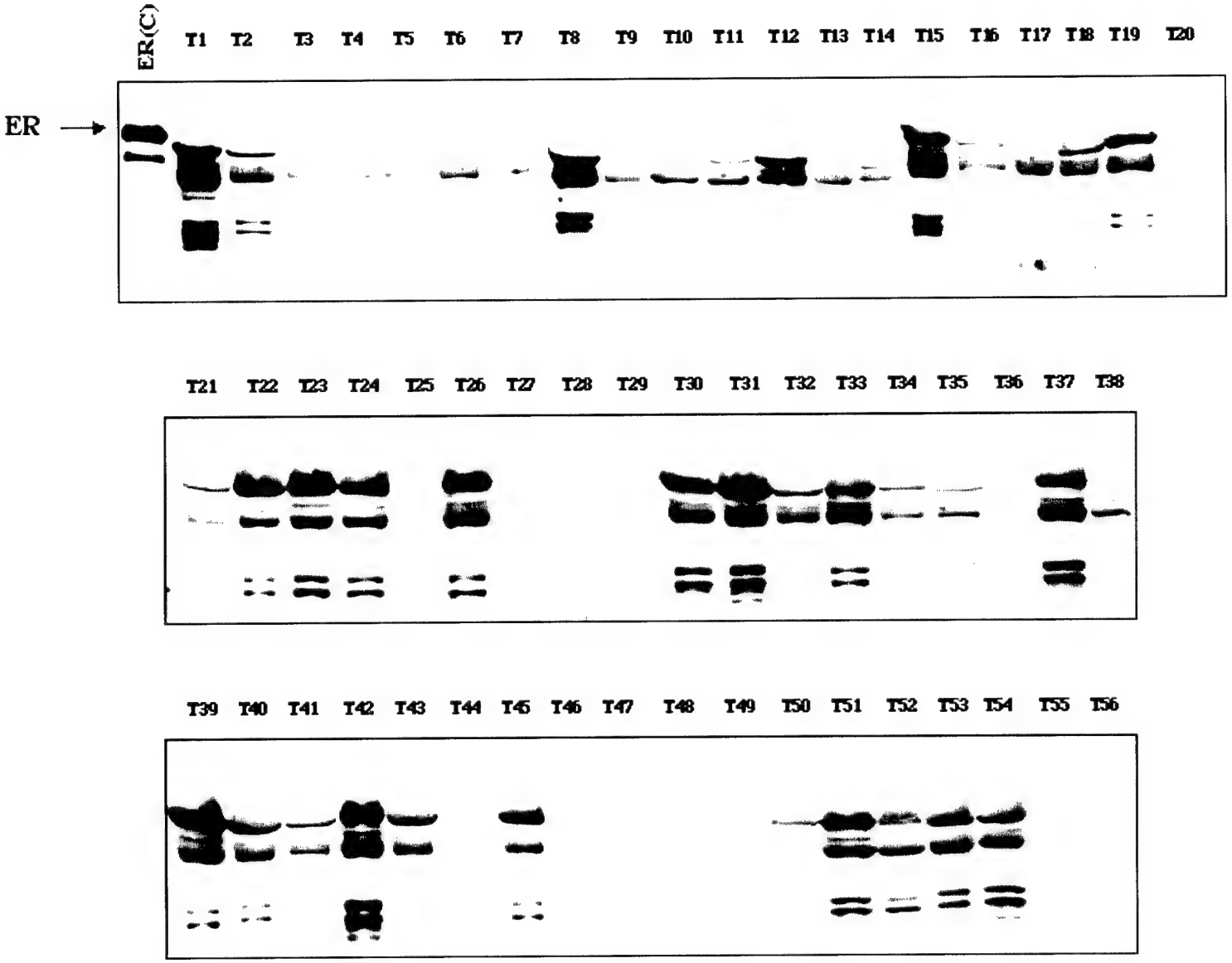

Figure 8: Expression analysis of ER-alpha in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of ER-alpha was analyzed by Western blot analysis using an anti-ER antibody. C, Purified ER protein was used as a control. T1-T56 represent different tumor samples.

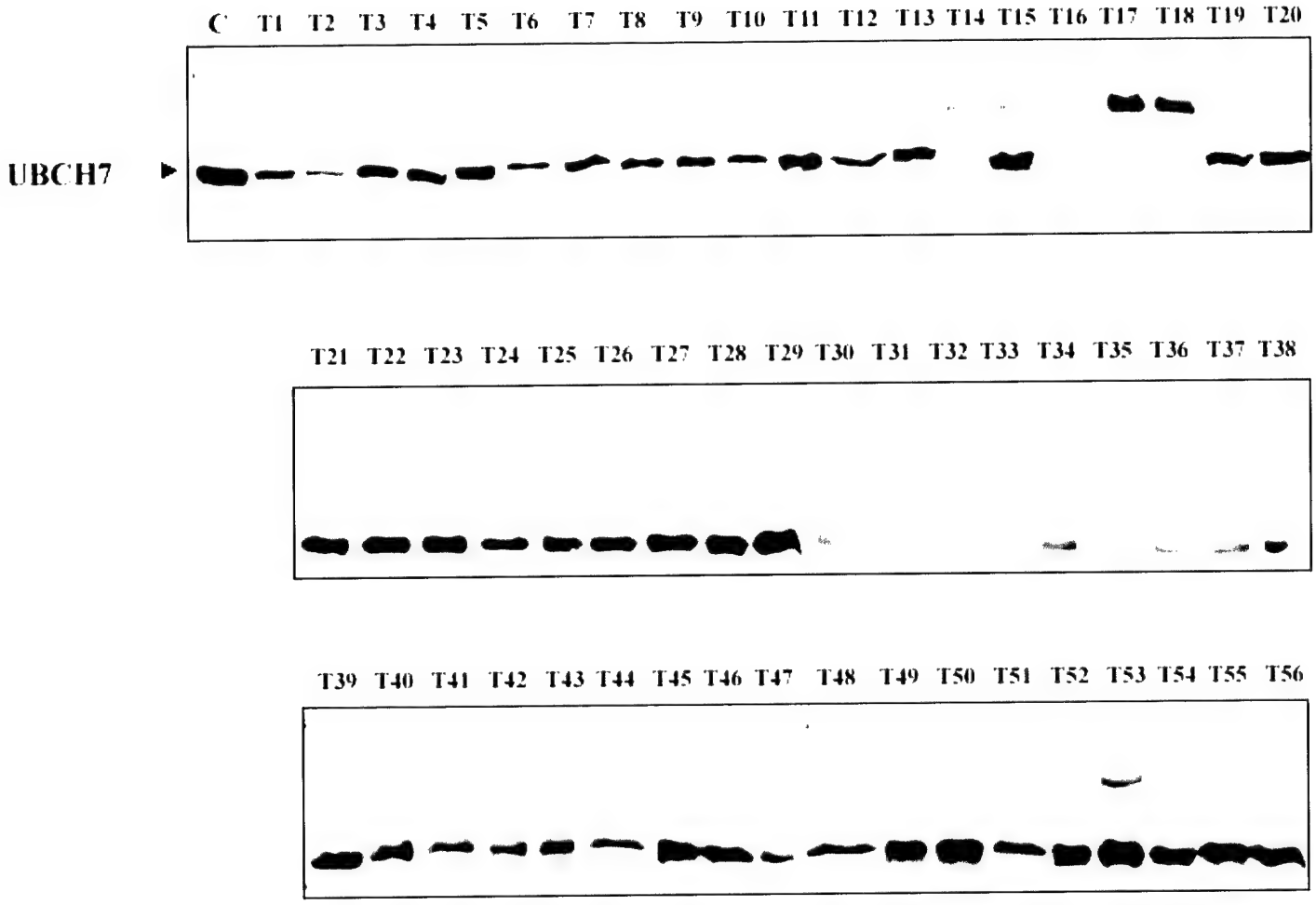

Figure 9: Expression analysis of UbcH7 in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of UbcH7 was analyzed by Western blot analysis using an anti-UbcH7 antibody. C, Purified UbcH7 protein was used as a control. T1-T56 represent different tumor samples.

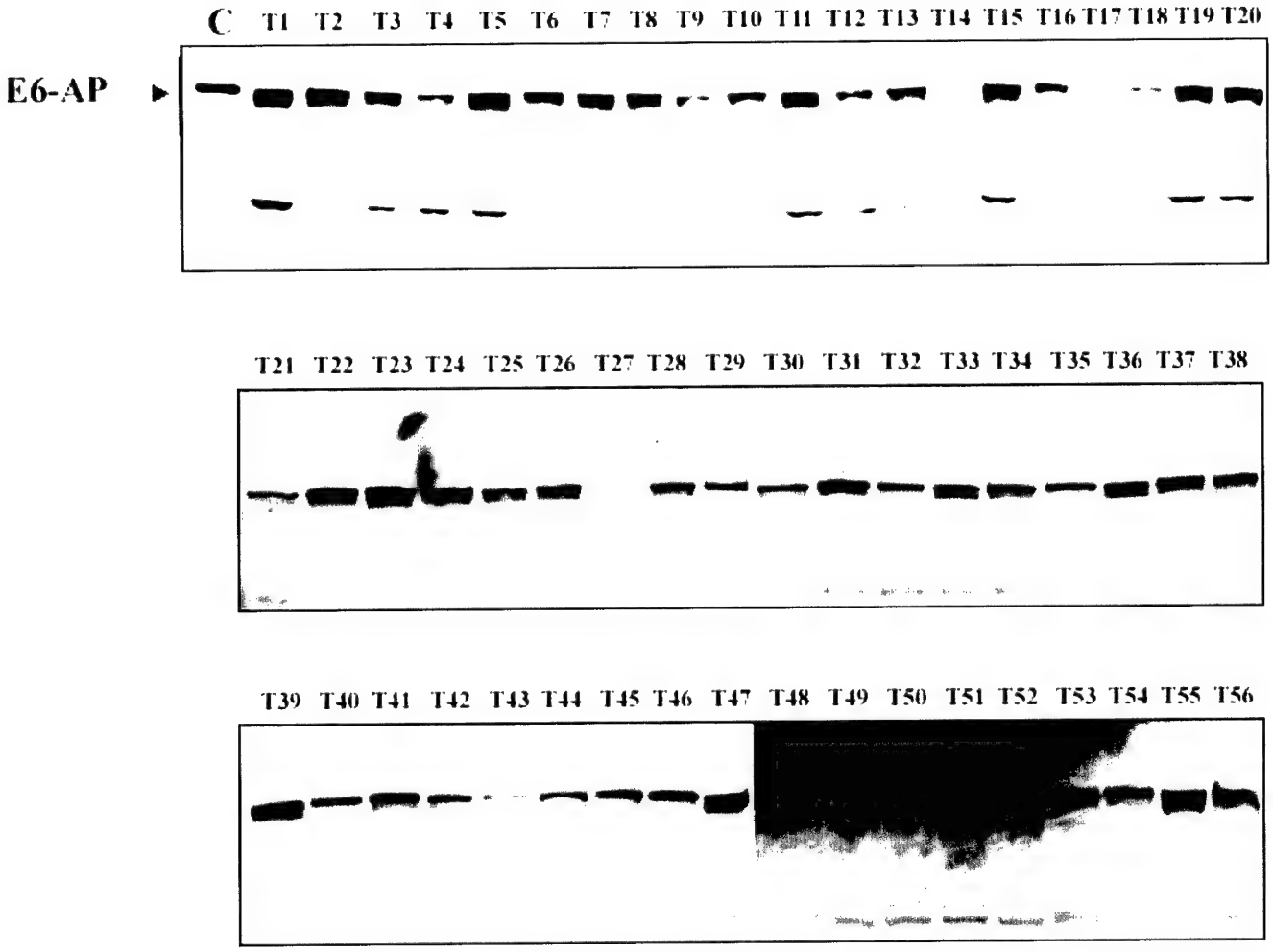

Figure 10: Expression analysis of E6-AP in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of E6-AP was analyzed by Western blot analysis using an anti-E6-APantibody. C, Purified E6-AP protein was used as a control. T1-T56 represent different tumor samples.

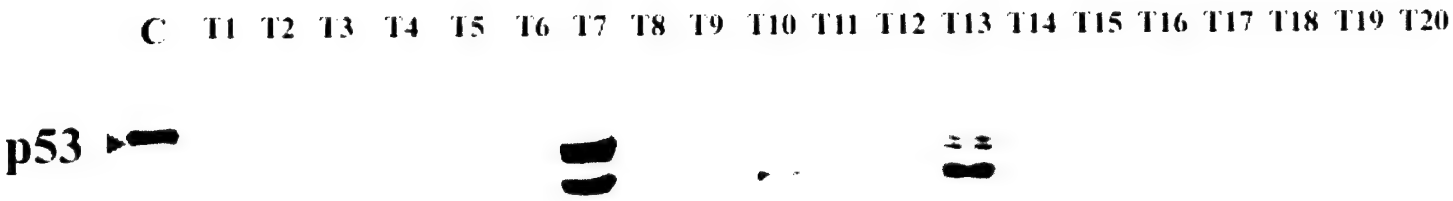

Figure 11: Expression analysis of p53 in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of p53 was analyzed by Western blot analysis using an anti-p53 antibody. C, Purified p53 protein was used as a control. T1-T20 represent different tumor samples.

Tumor #	E6-AP	ER	UbcH7	Tumor #	E6-AP	ER	UbcH7	Tumor #	E6-AP	ER	UbcH7
1	++++	++++	++	21	+	+	+++	41	++	+	+
2	+++	+	+	22	+++	++	+++	42	++	++++	+
3	++	-	+++	23	++++	++++	+++	43	+	++	+
4	+	-	+++	24	+++	+++	++	44	+	++	+++
5	++++	-/+	++++	25	++	-	+++	45	++	++	+++
6	++	-/+	+	26	++	++++	++	46	+++	-/+	+++
7	++	-	++	27	-/+	-/+	+++	47	+++	-/+	++
8	+++	+++	++	28	++	-/+	++++	48	+	-/+	++
9	+	-/+	++	29	++	-/+	++++	49	++	-/+	+++
10	++	-/+	++	30	++	+++	-/+	50	++++	+	+++
11	+++	+	++++	31	++++	++++	-/+	51	++++	+++	++
12	++	++	+++	32	++	+	-/+	52	++++	+	++++
13	++	-/+	+++	33	+++	++	-/+	53	+++	++	++++
14	-/+	-/+	-/+	34	+++	+	-/+	54	+++	++	+++
15	++++	++++	+++	35	++	+	-/+	55	++++	-/+	+++
16	++	-/+	-/+	36	+++	-	-/+	56	+++	-/+	+++
17	-/+	-	-/+	37	+++	++++	-/+				
18	+	+	-/+	38	+++	+	-/+				
19	++++	++	++	39	++++	+++	++				
20	++++	-	++++	40	+	++	+				

Figure 12: Correlation of the expression of E6-AP and UbcH7 with that of ER-alpha in breast tumors. Expression levels of E6-AP, ER-alpha and UbcH7 from Western blot analysis was artificially graded according to the density of the bands. "-" represents negative expression, whereas "-/+" represents very low expression. From "+" to "++++" represent the gradually increasing levels of expression from low to high. Sprearman Rank Correlation Coefficient for the expression of E6-AP with that of ER-alpha is 0.38, p=0.004. However, there is no correlation between UbcH7 and ER-alpha expression and UbcH7 and E6-AP expression.

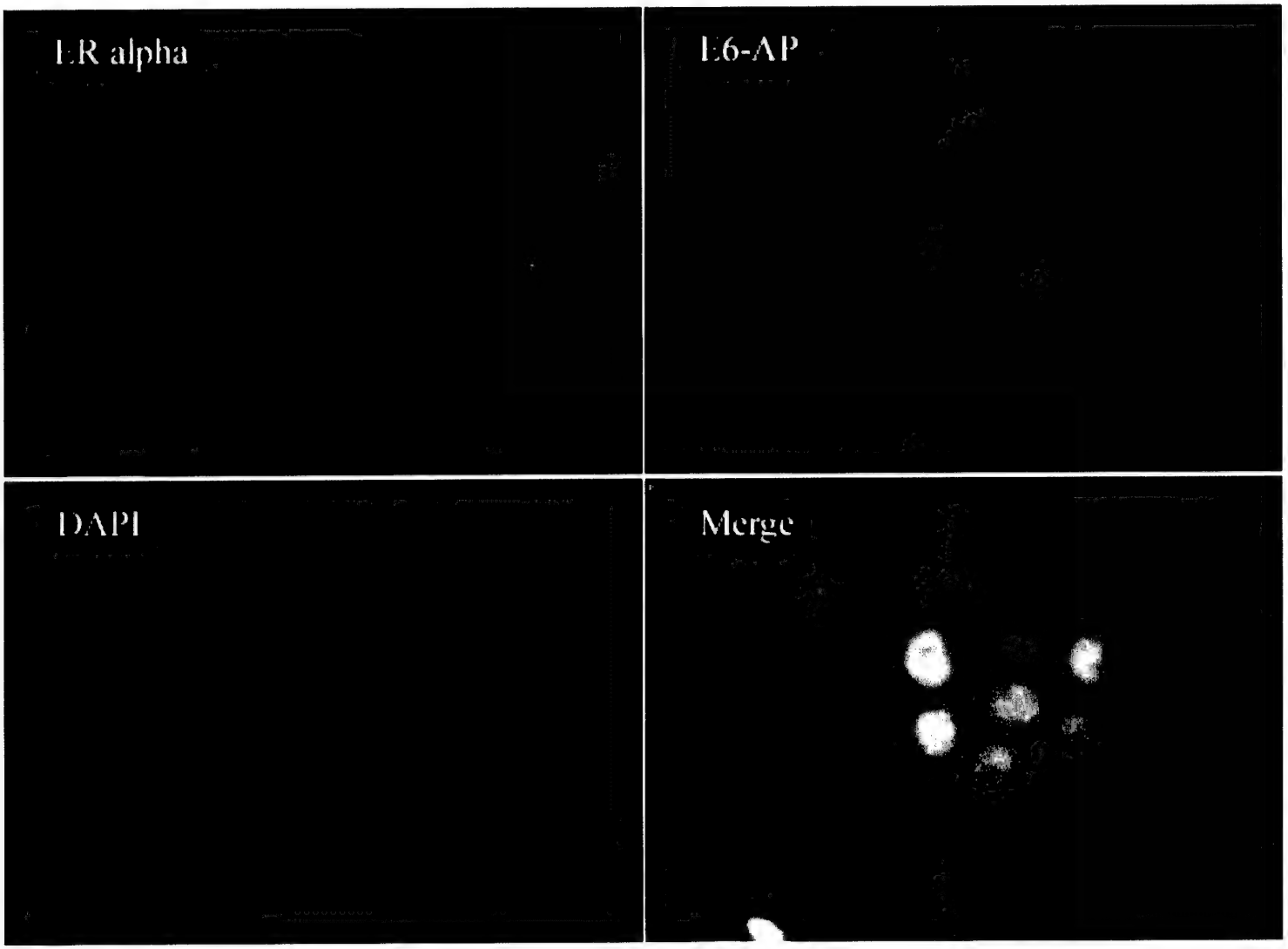

Figure 13: Colocalization of E6-AP and ER-alpha in T47D human breast cancer cell line by immunocytochemistry. Cells were grown on a chamber slide for 24 hours and the expression of endogenous E6-AP and ER-alpha was analyzed by fluorescent immunocytochemistry using either an anti-E6-AP or ER-alpha antibody. Positive signal for E6-AP is seen as (red) spots, ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. Yellow spots (merge) indicate colocalization of ER-alpha and E6-AP. ER-alpha, ER-alpha expression profile; E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

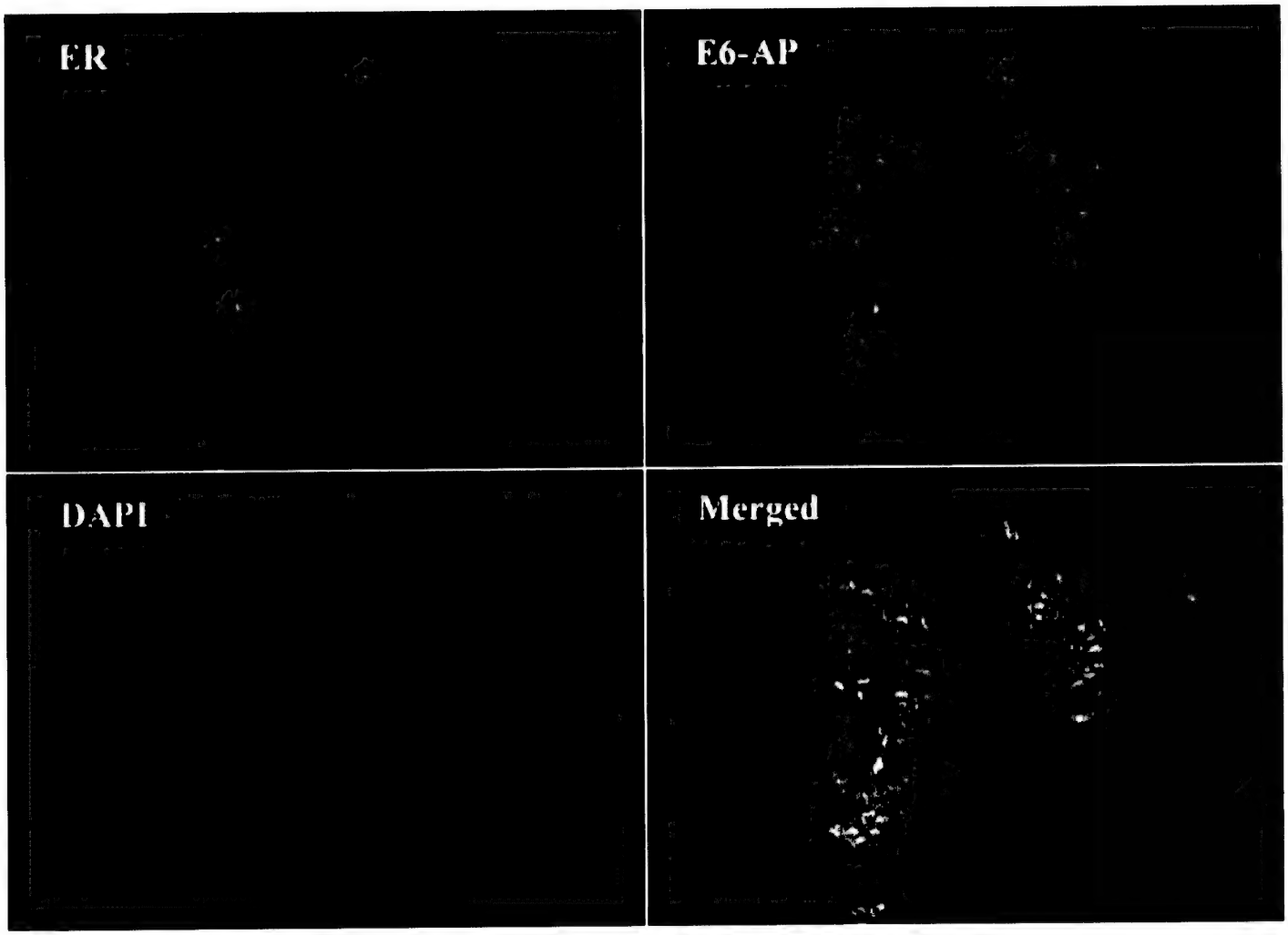

Figure 14: Expression analysis of E6-AP and ER-alpha in normal human breast tissues by immunocytochemistry. The expression of endogenous E6-AP and ER-alpha was analyzed by fluorescent immunocytochemistry using either an anti-E6-AP or ER-alpha antibody. Positive signal for E6-AP is seen as (red) spots, ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAPI staining. ER, ER-alpha expression profile; E6-AP, E6-AP expression profile; DAPI, DAPI staining for nucleus.

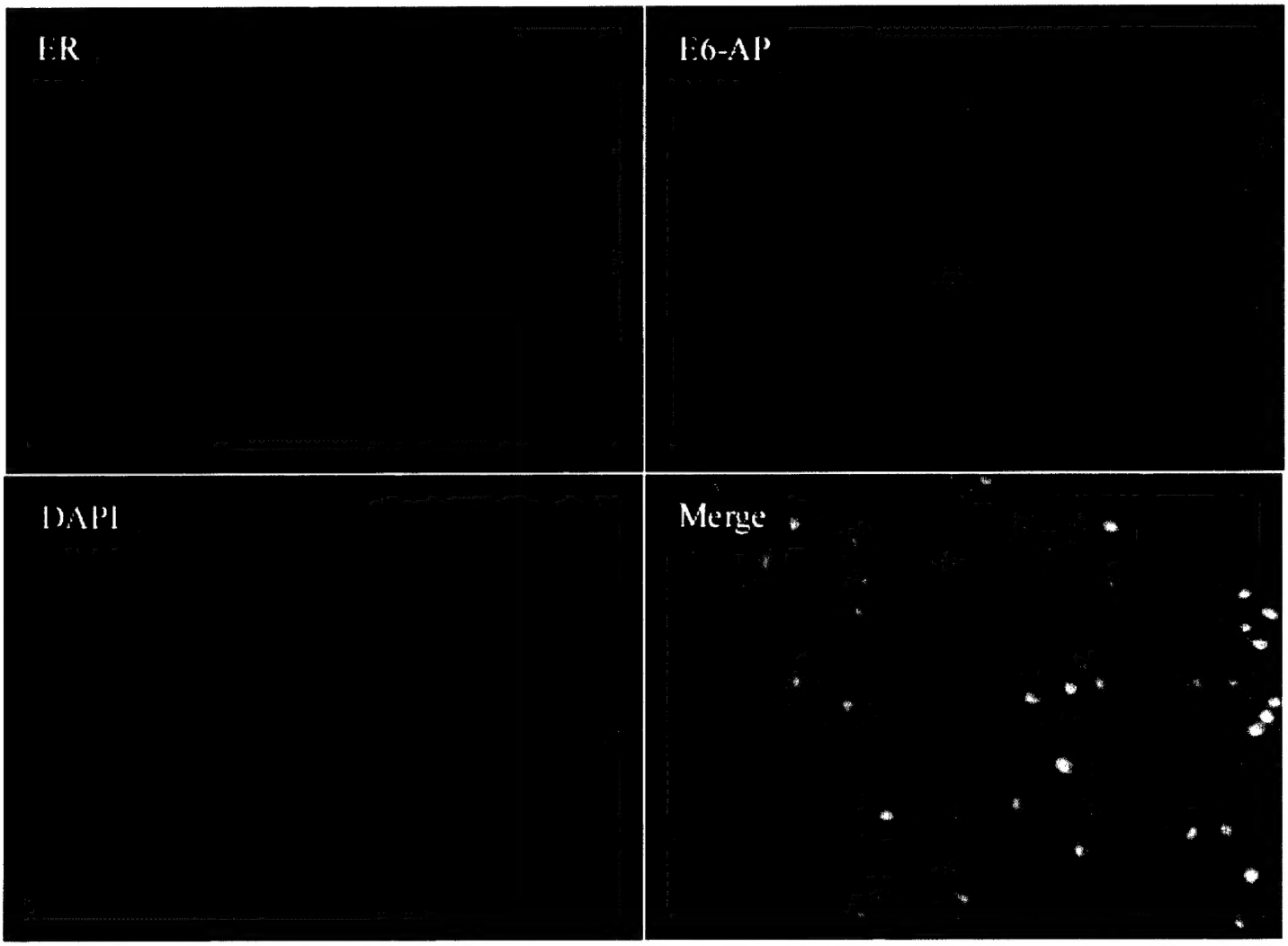

Figure 15: Expression analysis of E6-AP and ER-alpha in human breast tumors by immunocytochemistry. The expression of endogenous E6-AP and ER-alpha was analyzed by fluorescent immunocytochemistry using either an anti-E6-AP or ER-alpha antibody. Positive signal for E6-AP is seen as (red) spots, ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAPI staining. Yellow spots (merge) indicate coexpression of ER-alpha and E6-AP. ER, ER-alpha expression profile; E6-AP, E6-AP expression profile; DAPI, DAPI staining for nucleus.

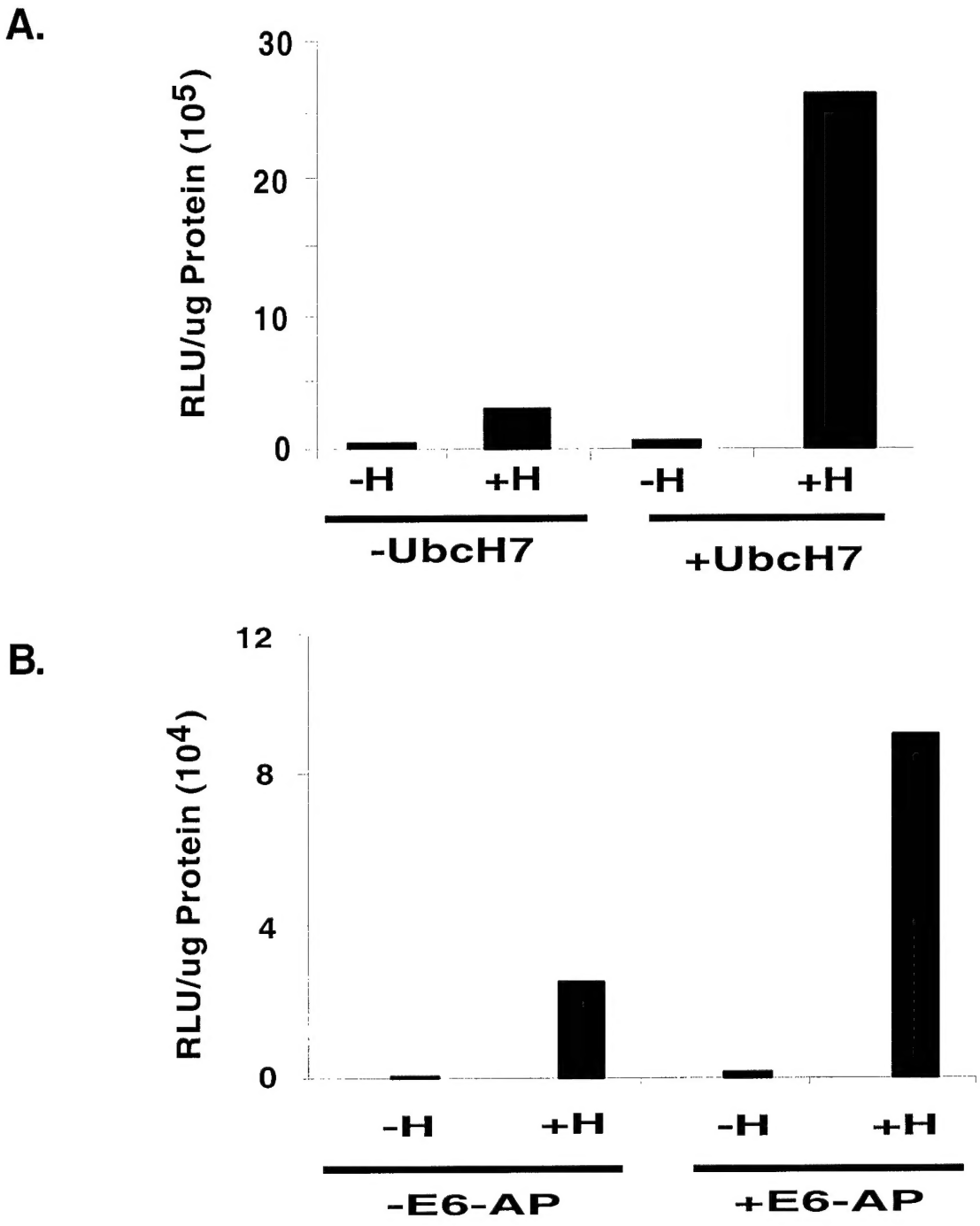

Figure 16: His-tagged UbcH7 and E6-AP were able to coactivate PR activity. Hela cells were transiently transfected with progesterone receptor expression plasmid and progesterone-responsive reporter plasmid in the absence or presence of his-tagged UbcH7 or E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M Progesterone (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

Appendix 2

Abstract

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Submission Type: General Submission - Poster Session
Category: Basic Science :: Hormones & Cancer - 09
Applying For Award: Travel Award
Sponsor: Zafar Nawaz
Character Count: 1951 (Max: 2500)
Keywords: tumorigenesis, coactivator, steroid receptor

Created: 30 Jan 2001 11:20 am
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Submitted: 2 Feb 2001 10:19 am

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INVOLVEMENT OF STEROID HORMONE RECEPTOR COACTIVATORS, E6-AP AND UBCS, IN THE DEVELOPMENT OF BREAST TUMORS.

X. Gao[1], G.M. Clark[2], F. Yan[1] and Z. Nawaz[1]. [1]Molecular and Cellular Biology, Baylor College of Medicine, Houston., TX, United States, [2]Breast Center, Baylor College of Medicine, Houston, TX, United States

Steroid hormones, estrogen and progesterone, are known to play a major role in the development of breast tumors by functioning through their cognate intracellular receptors, estrogen receptor (ER), and progesterone receptor (PR), respectively. Coregulators of steroid hormone receptors are important mediators of steroid receptors' function. Changes in the expression of these coactivators may contribute to mammary gland tumorigenesis. Recently, our laboratory identified several ubiquitin pathway enzymes, such as E6-associated protein (E6-AP) and ubiquitin conjugating enzymes (UBCs), as coactivators of steroid hormone receptors. Seperately, it was reported that E6-AP was overexpressed in a spontaneous mouse model of mammary gland tumorigenesis. To study the expression profiles of E6-AP and UBCs in human breast tumors, we examined 56 advanced stage human breast cancer biopsy samples. We found a correlation between the expression of E6-AP and the expression of ER-alpha in these breast tumors using Western blot analysis. The Spearman Rank Correlation Coefficient was 0.38 and the p value was 0.004, indicating that this correlation was statistically significant. Furthermore, the expression of E6-AP also correlated with that of UbcH7 (p=0.002), although the latter did not correlate with the expression of ER-alpha (p=-0.16). Our data provide the first evidence of a relationship between steroid hormone receptors and their coactivators, E6-AP and UBCs, suggesting a possible role of these coactivators in mammary gland tumorigenesis. It also indicates that E6-AP may be a potential target for breast cancer therapy.